# Elevated Glucose Changes the Expression of Ionotropic Glutamate Receptor Subunits and Impairs Calcium Homeostasis in Retinal Neural Cells

*Ana R. Santiago,*[1,2] *Susana C. Rosa,*[1] *Paulo F. Santos,*[1,3] *Armando J. Cristóvão,*[1,3] *Alistair J. Barber,*[4] *and António F. Ambrósio*[1,2]

**Purpose.** Altered glutamatergic neurotransmission and calcium homeostasis may contribute to retinal neural cell dysfunction and apoptosis in diabetic retinopathy (DR). The purpose of this study was to determine the effect of high glucose on the protein content of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate glutamate receptor subunits, particularly the GluR2 subunit, because it controls $Ca^{2+}$ permeability of AMPA receptor-associated channels. The effect of high glucose on the concentration of cytosolic free calcium ($[Ca^{2+}]_i$) was also investigated.

**Methods.** The protein content of GluR1, GluR2, GluR6/7, and KA2 subunits was assessed by Western blot. Cobalt staining was used to identify cells containing calcium/cobalt-permeable AMPA receptors. The $[Ca^{2+}]_i$ changes evoked by KCl or kainate were recorded by live-cell confocal microscopy in R28 cells and in primary cultures of rat retina, loaded with fluo-4.

**Results.** In primary cultures, high glucose significantly decreased the protein content of GluR1 and GluR6/7 subunits and increased the protein content of GluR2 and KA2 subunits. High glucose decreased the number of cobalt-positive cells, suggesting a decrease in calcium permeability through AMPA receptor-associated channels. In high-glucose–treated cells, changes in $[Ca^{2+}]_i$ were greater than in control cells, and the recovery to basal levels was delayed. However, in the absence of $Na^+$, to prevent the activation of voltage-sensitive calcium channels, the $[Ca^{2+}]_i$ changes evoked by kainate in the presence of cyclothiazide, which inhibits AMPA receptor desensitization, were significantly lower in high-glucose–treated cells than in control cultures, further indicating that AMPA receptors were less permeable to calcium. Mannitol, used as an osmotic control, did not cause significant changes compared with the control.

**Conclusions.** The results suggest that elevated glucose may alter glutamate neurotransmission and calcium homeostasis in the retina, which may have implications for the mechanisms of vision loss in DR. (*Invest Ophthalmol Vis Sci.* 2006;47:4130–4137) DOI:10.1167/iovs.06-0085

Diabetic retinopathy (DR) is the leading cause of new cases of blindness in working age adults in Western countries.[1] Although DR is usually considered a vascular disease, recent reports have clearly shown that the retinal neurons are also affected by diabetes (reviewed by Barber).[2] In fact, loss of color[3,4] and contrast sensitivity[5] have been reported as early symptoms of neuronal dysfunction of the retina, occurring within 2 years after the onset of diabetes in humans. More recently, it has been demonstrated that retinal neurons undergo apoptosis in diabetic rats, mice, and humans.[6–10]

Glutamate is the main excitatory neurotransmitter in the retina, and it is essential for the transmission of the visual signal from the photoreceptors to the bipolar cells and from these to the ganglion cells.[11] However, results in several studies suggest that glutamate is involved in the development of DR. The conversion of glutamate to glutamine is impaired in the retinas of diabetic rats,[12] and the concentration of glutamate is increased in the retinas and vitreous of patients with proliferative DR.[13,14] Moreover, we recently demonstrated that the release of D-aspartate evoked by membrane depolarization is increased in diabetic rat retinas and in retinal cell cultures exposed to high glucose,[15] indicating that diabetes can affect the mechanisms of glutamate release. Overall, these results show that the extracellular concentration of glutamate in the retina may be increased by diabetes, leading to an overactivation of glutamate receptors and consequent neuronal dysfunction.

Glutamate receptors can be divided into two distinct groups: the fast-acting ligand-gated ion channels and the slower-acting metabotropic receptors. Based on their physiological, pharmacologic, and molecular properties, the ionotropic glutamate receptors are further subdivided into three groups: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate (KA), and *N*-methyl-D-aspartate (NMDA) receptors. Whereas the metabotropic receptors are single polypeptides linked to G-proteins, the ionotropic receptors are multimeric, with AMPA receptors being assembled from subunits designated GluR1–4; kainate receptors from subunits GluR5–7, KA1, and KA2; and NMDA receptors from NR1, NR2A-D, and NR3A subunits. The ion channels associated with NMDA receptors are highly permeable to $Ca^{2+}$, whereas AMPA and kainate receptor-associated channels are usually less permeable to $Ca^{2+}$. However, certain combinations of subunits increase the $Ca^{2+}$-permeability of AMPA and kainate receptor-linked channels. For example, AMPA receptors lacking the GluR2 subunit are $Ca^{2+}$-permeable.[16–18]

Activation of ionotropic glutamate receptors leads to an increase in cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). If these receptors are overstimulated, the sustained increase in $[Ca^{2+}]_i$ can induce cell dysfunction and ultimately lead to cell death.[19]

From the [1]Center for Neuroscience and Cell Biology, Department of Zoology, the [2]Center of Ophthalmology of Coimbra, Institute of Biomedical Research on Light and Image (IBILI), Faculty of Medicine, and the [3]Department of Zoology, University of Coimbra, Coimbra, Portugal; and the [4]Department of Ophthalmology, The Penn State Retina Research Group, Penn State College of Medicine, Hershey, Pennsylvania.

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Corresponding author: António F. Ambrósio, Center of Ophthalmology of Coimbra, IBILI, Celas, 3000-548 Coimbra, Portugal; fambrosio@ibili.uc.pt.



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Excitotoxicity resulting from overstimulation of glutamate receptors has been implicated in other retinal diseases, including retinitis pigmentosa and glaucoma.[20,21] In diabetes, cumulative evidence shows that $Ca^{2+}$ homeostasis may be impaired, and defects in $Ca^{2+}$ regulation in several tissues, including the nervous system, have been reported (reviewed by Biessels et al.[22]). In dorsal horn neurons from streptozotocin (STZ)-induced diabetic rats, the activity of N- and L-voltage–sensitive $Ca^{2+}$ channels is substantially increased.[23,24] Furthermore, immunoreactivity for the $Ca^{2+}$ binding proteins, parvalbumin, and calbindin, is increased in amacrine and bipolar cells of the retina of diabetic rats, suggesting a response to increases in intracellular $Ca^{2+}$[25] and raising the possibility that diabetes alters $Ca^{2+}$ homeostasis of neurons in the retina.

This study investigated the effect of elevated concentrations of glucose on the protein content of GluR1, GluR2, GluR6/7, and KA2 subunits and the $[Ca^{2+}]_i$ changes in retinal neural cell cultures. A cobalt uptake staining technique was used to identify neurons that express calcium/cobalt-permeable AMPA receptor–associated channels, which lack the GluR2 subunit.[26,27] The effect of high glucose on the function of $Ca^{2+}$-permeable AMPA receptor–associated channels was also investigated by fluorescent $Ca^{2+}$-imaging, in the absence of $Na^+$ [replaced by *N*-methyl-D-glucamine (NMG)], which prevents membrane depolarization and therefore the opening of voltage-sensitive calcium channels. The cells were stimulated with kainate to cause sustained currents, potentiated by the drug cyclothiazide (CTZ), which inhibits AMPA receptor desensitization.[28,29] Because NMDA receptors were blocked by $Mg^{2+}$ in the medium, the stimulation with kainate in nondesensitizing conditions allowed $Ca^{2+}$ influx only through $Ca^{2+}$-permeable AMPA receptor–associated channels. Overall, the data demonstrate that an elevated concentration of glucose alters the glutamate receptor subunit content and $Ca^{2+}$ homeostasis in retinal neurons, which could account for some of the functional changes observed in DR.

## METHODS

### Materials

Fetal bovine serum was purchased from BioWhittaker (Walkersville, MD) and newborn calf serum was purchased from HyClone (Logan, UT). Trypsin (USP grade) was purchased from Invitrogen Corp. (Carlsbad, CA). Kainate (KA), cyclothiazide (CTZ), and 4-methylglutamate were purchased from Tocris (Ellisville, MO). LY 303070 was a kind gift of Lilly Research Laboratories (Indianapolis, IN). The acetoxymethyl ester of fluo-4 (fluo-4/AM) was purchased from Invitrogen-Molecular Probes (Eugene, OR). Complete miniprotease inhibitor cocktail tablets were purchased from Roche (Basel, Switzerland). Concanavalin A and the antibody anti-MAP2 were from Sigma-Aldrich (St. Louis, MO), and the antibodies anti-GluR1, anti-GluR6/7, and anti-KA2 were from Upstate (Charlottesville, VA). The antibody anti-GluR2 was from BD Biosciences (San Jose, CA). The polyvinylidene difluoride (PVDF) membranes, the alkaline phosphatase-linked anti-rabbit secondary antibodies and the enhanced chemiluminescence (ECL) reagent were obtained from GE Healthcare (Buckinghamshire, UK). Other reagents used in immunoblot experiments were purchased from Bio-Rad (Hercules, CA). All other reagents were from Sigma-Aldrich or from Merck KGaA (Darmstadt, Germany).

### Primary Culture of Rat Retinal Neural Cells

All procedures involving animals were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinal cell cultures were obtained from 3- to 5-day-old Sprague-Dawley rats. Briefly, rat pups were anesthetized by intraperitoneal injection of ketamine (0.05 mL of 100 mg/mL) before death by rapid decapitation. Retinas were dissected in sterile $Ca^{2+}$- and $Mg^{2+}$-free Hanks' balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.45 $KH_2PO_4$, 0.34 $Na_2HPO_4$, 4 $NaHCO_3$, and 5 glucose [pH 7.4]) with a dissection microscope. The retinas were digested with 0.1% trypsin (wt/vol) for 15 minutes at 37°C. After digestion, the cells were collected by centrifugation (1 minute, 140 *g*) and resuspended in Eagle's minimum essential medium (MEM), containing 5 mM glucose supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 $\mu$g/mL). The cells were plated at a density of $2 \times 10^6$ cells/cm$^2$ on poly-D-lysine substrate (0.1 mg/mL). For immunoblot analysis, cells were plated on 60-mm plastic Petri dishes. For immunohistochemistry, cells were plated on glass coverslips. For $Ca^{2+}$ imaging studies, cells were plated on 35-mm glass-bottomed culture dishes (MatTek; Ashland, MA). The cells were maintained at 37°C in a humidified incubator with 5% $CO_2$/air. After 2 days in culture, cells were incubated with 25 mM D-glucose, yielding a total 30 mM glucose. For osmotic control, cells were incubated with 25 mM D-mannitol (plus 5 mM glucose in the culture medium) and maintained for a further 7 days. The concentration of glucose in the control condition was 5 mM.

### R28 Cell Culture

The R28 cell line originated from a mixed population of retinal cells transfected with E1A.NR.3, followed by selection of neuronlike clones, was a generous gift from Gail Seigel (Ross Eye Institute, SUNY at Buffalo, NY).[30,31] It has been shown that R28 cells grown with laminin and cAMP have a neuronlike phenotype.[32] The cells were plated at a seeding density of $1.28 \times 10^5$ cells/cm$^2$, in Dulbecco's modified Eagle's medium (DMEM) containing 5 mM glucose, supplemented with 10% newborn calf serum and 250 $\mu$M cAMP, on laminin-coated (1 mg/mL) glass-bottomed culture dishes. The cells were maintained at 37°C in a humidified atmosphere of 5% $CO_2$/air. For experiments, cells were incubated with 15 mM D-glucose yielding a total 20 mM glucose, or for osmotic control, with 15 mM D-mannitol (plus 5 mM glucose), and cultures were kept for 48 to 72 hours. The concentration of glucose in the control condition was 5 mM.

### Western Blot Analysis of AMPA and Kainate Receptor Subunits

Cells were washed twice with cold phosphate-buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 10 $Na_2HPO_4$, and 1.8 $KH_2PO_4$ [pH 7.4]) and lysed in 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS supplemented with complete miniprotease inhibitor cocktail tablets. The protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA), and 15 $\mu$g protein (when blotting for GluR1 and GluR6/7) or 60 $\mu$g protein (when blotting for GluR2 and KA2) from each sample were used for immunoblots, after adding 6× concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M dithiothreitol [DTT], 0.012% bromophenol blue) and heating the samples for 5 minutes at 95°C. Proteins were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred electrophoretically to polyvinylidene (PVDF) membranes. The membranes were blocked for 1 hour at room temperature in Tris-buffered saline (in mM: 137 NaCl and 20 Tris-HCl [pH 7.6]) containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk. Incubation with the primary antibodies (anti-GluR1, 1:500; anti-GluR2, 1:500; anti-GluR6/7, 1:500; and anti-KA2, 1:600) was performed overnight at 4°C. After washing for 1 hour in TBS-T with 0.5% low-fat milk, the membranes were incubated for 1 hour at room temperature with an alkaline phosphatase-linked secondary antibody (anti-rabbit IgG 1:20,000 in TBS-T with 1% low fat milk). The membranes were processed for detection of ionotropic glutamate receptor subunits using the ECF system on a gel imager (Versa Doc Imaging System; Bio-Rad), and digital quantification was performed (Quantity One; Bio-Rad).

### Cobalt Staining

Cobalt staining was performed as described previously,[16,33] with some modifications. The cells were washed twice with HEPES buffer (in mM:

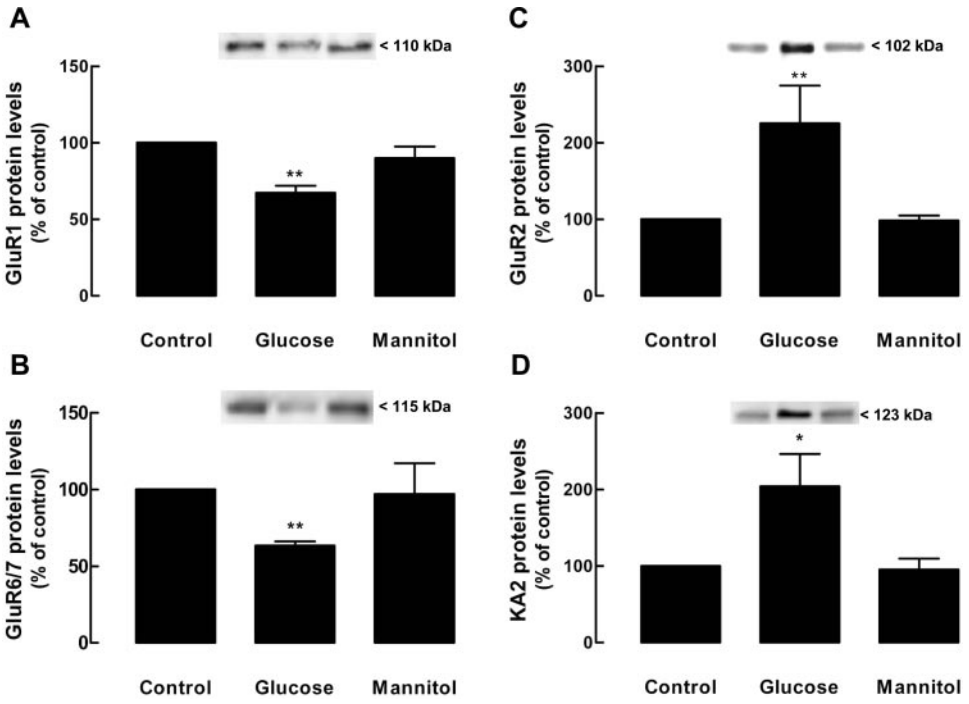


**FIGURE 1.** High glucose altered the protein levels of AMPA and kainate receptor subunits in primary cultures. Primary cell cultures from rat retina were incubated in 5 mM glucose (Control), 30 mM glucose (Glucose), or 25 mM mannitol plus 5 mM glucose (Mannitol) for 7 days. Total cell extracts of cultured retinal neural cells were assayed for (**A**) GluR1, (**B**) GluR2, (**C**) GluR6/7, or (**D**) KA2 subunit proteins. Representative Western blots are presented above the graphs for each antibody tested. Densitometry was performed on each band, and the results are expressed as the percentage of control ± SEM of results in at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control; one-way ANOVA followed by the Dunnett post hoc test.

132 NaCl, 4 KCl, 0.7 $MgCl_2$, 0.5 $CaCl_2$, 6 glucose, and 20 HEPES [pH 7.4]) at 37°C, and then stimulated with 100 $\mu$M KA in the presence of 30 $\mu$M CTZ (to prevent AMPA receptor desensitization) and 5 mM $CoCl_2$, for 30 minutes. After stimulation, the cells were washed twice with HEPES buffer supplemented with 2 mM EDTA to remove excess extracellular $Co^{2+}$. Intracellular $Co^{2+}$ was precipitated with 0.12% $Na_2S$ in HEPES buffer for 5 minutes. The cells were then washed with HEPES buffer and fixed with 4% paraformaldehyde containing 4% sucrose in PBS for 30 minutes at room temperature. CoS precipitates were enhanced by incubation in hot (50°C) developing buffer (in mM: 292 sucrose, 15.5 hydroquinone, and 42 citric acid), containing 1 mg/mL $AgNO_3$, for 1 hour in the dark. Staining was stopped by washing cells with hot developing buffer. To preserve the stain, we incubated the cells with 5% sodium thiosulfate at room temperature for 5 minutes. The cells were rinsed twice in PBS followed by washing with distilled water, air dried for 24 hours, and then mounted (Entellan; Merck). The preparations were visualized with a microscope (Axiovert 200; Carl Zeiss Meditec, Inc.) coupled to a photomicrograph system (Cool Snap HQ digital camera; Roper Scientific, Tucson, AZ). Images of five to seven arbitrary fields from each coverslip were obtained, and the length of the processes stained with cobalt was measured with image-analysis software (Optimas 6.2; Media Cybernetics, Silver Spring, MD).

## Immunocytochemistry

The cells were washed twice with PBS, fixed with 1% paraformaldehyde for 10 minutes at room temperature, rinsed twice in PBS, and permeabilized with 1% Triton X-100 in PBS for 5 minutes. After blocking for 1 hour with 10% donkey serum plus 0.1% Triton X-100 in PBS, the cells were incubated with the primary antibody (anti-MAP2, 1:100, or anti-GluR2, 1:250) for 90 minutes. The cells were rinsed three times with the blocking solution, incubated with a Cy2-conjugated secondary antibody (donkey anti-mouse IgG, 1:1000), were rinsed, and mounted (Aqua Poly/Mount; Polysciences, Inc., Warrington, PA). The preparations labeled with the anti-MAP2 antibody were visualized with a confocal microscope (488 nm excitation, 495–532 nm emission band; TCS SP2 AOBS; Leica, Deerfield, IL), and five random fields were photographed for each condition. The preparations labeled with anti-GluR2 antibody were visualized with a microscope (Axioshop 2 Plus; Carl Zeiss Meditec, Inc.), coupled to a camera (Axiocam HRc; Carl Zeiss Meditec., Inc.). For each condition at least five random fields were photographed and the densitometry measurements were performed by delineating the cell bodies using the public-domain ImageJ program (http://rsb.info.nih.gov/ij/ developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

## Live-Cell Confocal Measurement of Intracellular Calcium

Primary cultures and R28 cells, plated on 35-mm glass-bottomed dishes, were loaded with 5 $\mu$M fluo-4/AM, for 20 minutes at 37°C, in saline buffer (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1.4 $CaCl_2$, 6 glucose, and 10 HEPES [pH 7.4]). After they were rinsed the cells were placed on the stage of the inverted confocal microscope (63× oil immersion objective; TCS SP2 AOBS; Leica). Fluo-4 was excited with a 488-nm laser and emission was detected at 520 to 600 nm. Time-lapse images were acquired every 1.6 seconds. After 5 to 10 baseline images were acquired, the cells were stimulated with KCl or kainate. Image acquisition continued for 100 to 300 seconds. Image analysis was performed with the confocal microscope software. Briefly, regions of interest were defined by drawing an outline around each cell body, and the mean fluorescence was extracted across the time-lapse sequence of images to obtain fluorescence versus time plots for each cell. Background fluorescence was obtained from a region with no cells for every field examined and subtracted from the mean fluorescence. The mean fluorescence was also corrected for the mean baseline fluorescence determined before stimulation of the cells.

## Data Analysis

Data are expressed as the mean ± SEM. Statistical significance was determined by analysis of variance (ANOVA), followed by the Dunnett post hoc test.

## RESULTS

The effect of high glucose on the protein levels of AMPA (GluR1 and GluR2) and kainate (GluR6/7 and KA2) ionotropic glutamate receptor subunits was investigated, giving particular attention to the GluR2 subunit, because it controls the $Ca^{2+}$ permeability of AMPA receptor-associated channels. The effect

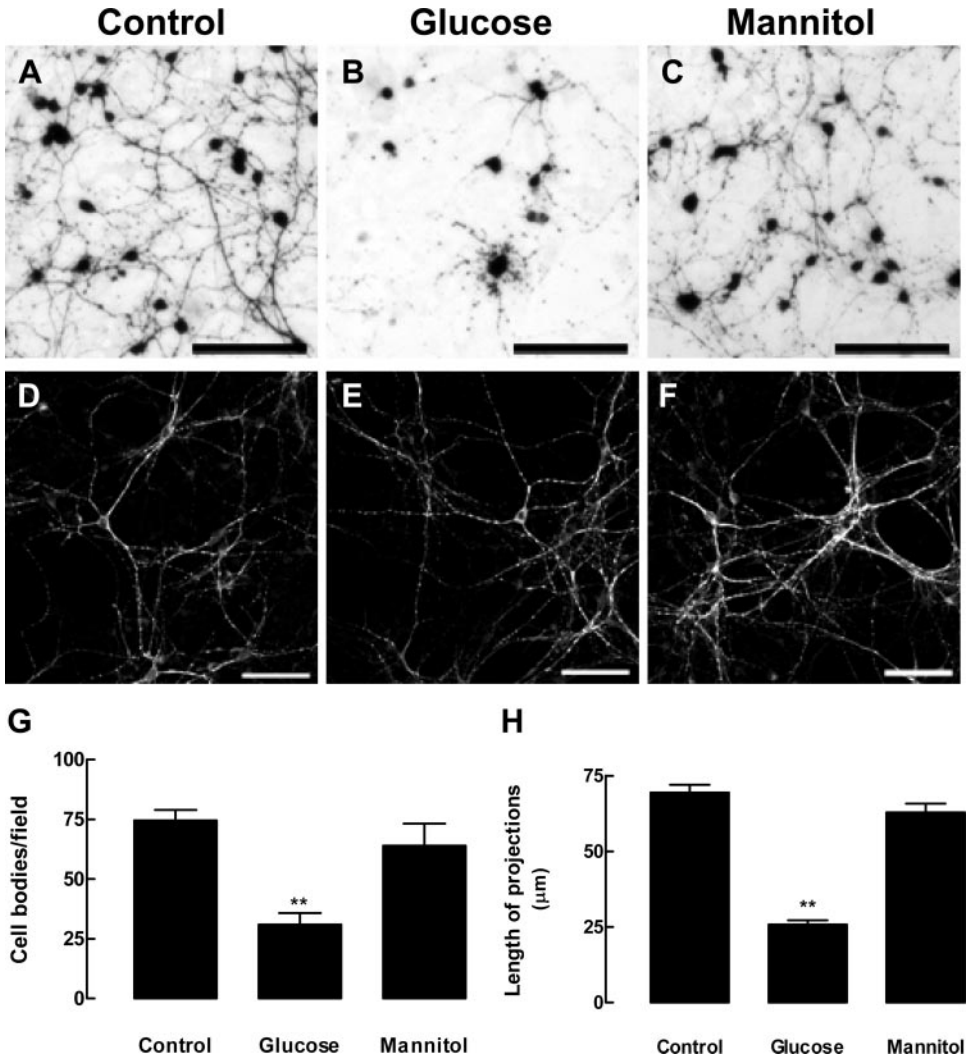


**FIGURE 2.** High glucose levels reduced $Co^{2+}$ uptake through AMPA receptors. Rat primary cell cultures were incubated in 5 mM glucose (Control), 30 mM glucose (Glucose), or 25 mM mannitol plus 5 mM glucose (Mannitol) for 7 days. The cells were stimulated with 100 $\mu$M KA under nondesensitizing conditions (with CTZ, 30 $\mu$M), in the presence of $Co^{2+}$. (**A**) In control cells, there was cobalt staining in both cell bodies and processes, (**B**) in high-glucose–treated cells there were fewer cell bodies and less cell processes stained with cobalt, (**C**) and in mannitol-treated cells cobalt staining was similar to that in control cells. (**D–F**) Cells were labeled for MAP2 by immunocytochemistry. There was no difference in the morphology of cells in any of the treatment groups. (**G**) The number of cell bodies stained with cobalt was significantly reduced in glucose-treated cells compared with the control cells. (**H**) The length of processes stained with cobalt was significantly reduced in high-glucose–treated cells compared with the control. Results shown in (**G**) and (**H**) are expressed as the mean ± SEM combined from results in four independent experiments. The images are representative of those obtained from four independent experiments performed in duplicate. $^{**}P < 0.01$; one-way ANOVA followed by the Dunnett post hoc test. Bar, 40 $\mu$m.

of high glucose on changes in $[Ca^{2+}]_i$ evoked by membrane depolarization or AMPA receptor activation was also studied in primary cultures and R28 cells.

## Alteration of the Protein Levels of Ionotropic Glutamate Receptor Subunits

The effect of high glucose on the expression of AMPA (GluR1 and GluR2) and kainate (GluR6/7 and KA2) receptor subunits was examined by immunoblot analysis of primary cell cultures from rat retina. In cells exposed to 30 mM glucose the protein content for the AMPA subunit, GluR1, was significantly less than control (67.3% ± 4.6% of the control, Fig. 1A), whereas that of GluR2 was significantly greater than the control (225.5% ± 49.1% of the control, Fig. 1B). The protein content of the GluR6/7 subunits was significantly less than the control (67.3% ± 4.6% of the control, Fig. 1C). In contrast, the protein content of the KA2 subunit in cells exposed to 30 mM glucose was significantly greater than the control (204.3% ± 42.3% of the control, Fig. 1D). The protein content of the glutamate receptor subunits in cells exposed to 25 mM mannitol (+5 mM glucose), which was used as the osmotic control, were not significantly different from the control (5 mM glucose).

## Effect of High Glucose on $Ca^{2+}$-Permeable AMPA Receptors

Because high glucose increased the content of the GluR2 subunit, this may reduce the $Ca^{2+}$ permeability of the AMPA receptor-associated channel. The cobalt staining technique was used to assess the effect of high glucose on the uptake of $Ca^{2+}$ through AMPA receptor-associated channels.[26,27] In control cultures (5 mM glucose), many cell bodies and processes were stained with cobalt (Fig. 2A). In contrast, cell cultures exposed to 30 mM glucose had fewer cells stained with cobalt, compared with the control (Fig. 2B). The number of cobalt-positive cells in cultures incubated with mannitol was not different from the control (Fig. 2C). In addition, the processes of neurons were heavily stained in control and mannitol-treated cells, whereas in the cells incubated with high glucose, most of the staining was confined to the cell bodies.

To ensure that the reduction in cobalt staining was not due to a change in morphology, cells were labeled with a monoclonal antibody to microtubule-associated protein 2 (MAP2), a cytoskeletal marker expressed only in neurons.[34] Neuronal projections and cell bodies were immunoreactive for MAP2 in all three culture conditions, and cell morphology was not influenced by higher concentrations of glucose or mannitol (Figs. 2D–F). In addition, the average length of the MAP2-immunoreactive processes was not significantly changed in the three conditions (data not shown). The average number of cell bodies stained with cobalt was significantly less in the high-glucose–treated cells compared with the control (Fig. 2G). The average length of cobalt-stained processes was also measured by digital image analysis. In high-glucose–treated cells the stained processes were significantly shorter than those of the

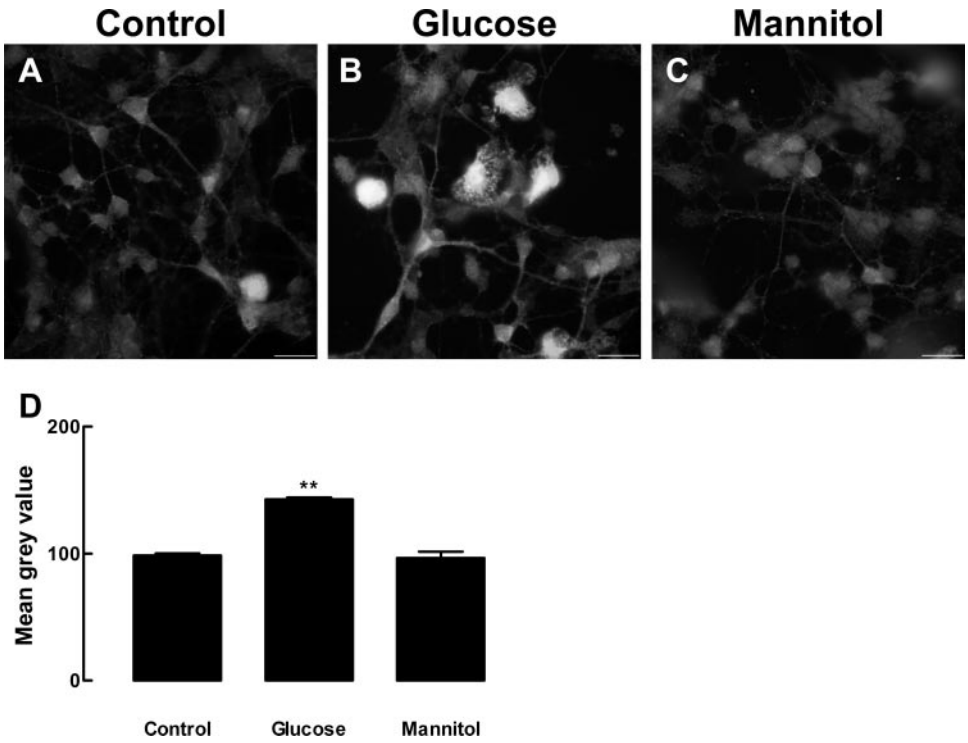


**FIGURE 3.** High glucose increased GluR2 immunoreactivity. Rat primary cell cultures were incubated in 5 mM glucose (Control), 30 mM glucose (Glucose), or 25 mM mannitol plus 5 mM glucose (Mannitol) for 7 days and labeled for GluR2 by immunocytochemistry. (**A**) Control cells were immunoreactive for GluR2 in both cell bodies and processes, (**B**) in high-glucose–treated cells GluR2 subunit immunoreactivity was enhanced compared with the control; (**C**) the immunoreactivity in mannitol-treated cells was essentially identical with that in control cells. (**D**) The mean gray value of GluR2 immunoreactivity, measured by image analysis was significantly greater in high-glucose–treated cells than in the control cultures. The images are representative of those obtained from three independent experiments. Results shown in (**D**) are expressed as the mean ± SEM obtained from five random fields in three independent experiments. $^{**}P < 0.01$; one-way ANOVA followed by the Dunnett post hoc test. Bar, 20 $\mu$m.

control, and in mannitol-treated cells there were no significant changes compared with the control (Fig. 2H).

The presence of $Ca^{2+}$-permeable kainate receptor-associated channels was also investigated by $Co^{2+}$ staining in cultured retinal neural cells. The selective activation of kainate receptors with 10 $\mu$M 4-methylglutamate or with 3 $\mu$M kainate did not result in $Co^{2+}$ staining (data not shown). The failure of kainate receptor activation to induce $Co^{2+}$ staining was not due to kainate receptor desensitization, because in the presence of 250 $\mu$g/mL concanavalin A, which inhibits kainate receptor desensitization, neither agonist caused $Co^{2+}$ staining (data not shown). In addition, stimulation of cells with 100 $\mu$M kainate in the presence of LY 303070 (15 $\mu$M), which selectively blocks AMPA receptors, did not cause any $Co^{2+}$ staining, indicating that staining was due to entry of $Co^{2+}$ through $Ca^{2+}$-permeable AMPA receptors. These results indicate that the ion channel associated with AMPA receptors is less permeable to $Ca^{2+}$ in high-glucose–treated cells, than the control cultures.

The decrease in cobalt uptake in high-glucose–treated cells may be due to an increased content of the GluR2 subunit. To examine this possibility, control cells and cells incubated with 30 mM glucose or 25 mM mannitol, were labeled by immunocytochemistry for the GluR2 subunit (Fig. 3). In control cells, GluR2 immunoreactivity was distributed throughout the cell bodies and processes (Fig. 3A). In most of the high-glucose–treated cells, the GluR2 immunoreactivity was enhanced, confirming that the GluR2 subunit was more abundant in these cells (Fig. 3B). GluR2 immunoreactivity in mannitol-treated cells was similar to control (Fig. 3C). Image analysis confirmed the significant increase in immunoreactivity in the high-glucose–treated cells compared with control and mannitol treatment (Fig. 3D).

## Effect of High Glucose on $[Ca^{2+}]_i$ Changes

Calcium is a key mediator of many intracellular processes, and its deregulation can cause malfunction, leading to apoptosis.[35] Voltage-sensitive $Ca^{2+}$ channels are the most important pathway for the elevation of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), and their opening is dependent on the membrane potential. On activation, ionotropic glutamate receptors also contribute to the increase in $[Ca^{2+}]_i$. Therefore, the effect of high glucose on changes in $[Ca^{2+}]_i$ evoked by membrane depolarization or activation of AMPA and kainate receptors was studied by confocal microscopy on live cells loaded with the fluorescent membrane-permeable $Ca^{2+}$ probe, fluo-4/AM. R28 cells were used as another model of retinal neurons. After stimulation with 20 mM KCl, the fluorescence intensity in control cells increased transiently and returned to baseline after 65 seconds (Figs. 4A, 4B). In cells grown in 20 mM glucose, the baseline fluorescence was similar to that in control cells (Fig. 4C). Membrane depolarization with KCl induced a significantly higher increase in the fluorescence, compared to control, and after 65 seconds the intensity in fluorescence was not similar to basal levels (Fig. 4D). Fluorescence intensity in high-glucose–treated cells did not recover to basal levels until the end of the experiment. As with control cells, in mannitol-treated cells the fluorescence was transiently increased by KCl and then returned to baseline (Fig. 4E). The effect of high glucose on the $[Ca^{2+}]_i$ changes evoked by either 20 mM KCl, kainate (100 $\mu$M) or kainate plus 30 $\mu$M CTZ (in $Na^+$-free medium), was also analyzed in primary cell cultures using fluo-4/AM. As observed in R28 cells, the fluorescence intensity evoked by 20 mM KCl in cells exposed to 30 mM glucose was significantly greater than in control cells (Fig. 5A). Moreover, high-glucose–treated cells did not recover to basal levels during the experiment. The fluorescence intensity in cells incubated with mannitol was not significantly different from that in control cells. Stimulation of primary cultures with 100 $\mu$M KA produced results similar to those obtained with KCl (Fig. 5B).

The influx of $Ca^{2+}$ through AMPA receptor–associated channels was examined by stimulating primary cultures with kainate in the presence of CTZ, which blocks desensitization of AMPA receptors, in a $Na^+$-free medium, in which $Na^+$ ions were replaced by NMG. This molecule does not cross cell membranes and thus prevents membrane depolarization and

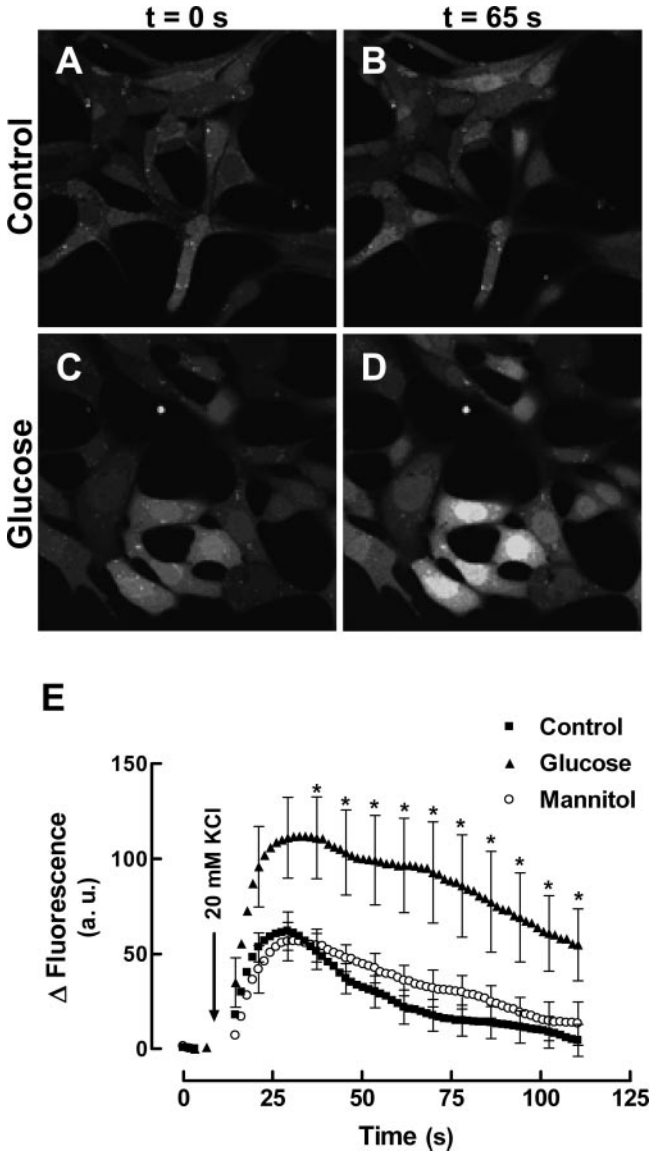


**FIGURE 4.** Increases in $[Ca^{2+}]_i$ after membrane depolarization were higher in R28 cells exposed to high glucose. R28 cells were grown in 5 mM glucose (Control) or 20 mM glucose (Glucose) for 2 days, loaded with fluo-4/AM for 20 minutes, and stimulated with 20 mM KCl. Changes in fluorescence were recorded by live-cell confocal microscopy. (**A**, **B**) In control cells, the fluorescence increased after stimulation with KCl and returned to baseline after 65 seconds. (**C**) In cells treated with high glucose the baseline fluorescence was similar to the control, and (**D**) the intensity of fluorescence 65 seconds after stimulation was higher in high-glucose–treated cells than in control cells. (**E**) The $[Ca^{2+}]_i$ changes were significantly higher in high-glucose–treated cells than in the control and mannitol-treated cells. Results are presented as the mean ± SEM of results in at least four independent experiments and represent changes in arbitrary fluorescence units over basal fluorescence, recorded before applying KCl. $^*P < 0.05$, significantly different from control; one-way ANOVA followed by the Dunnett post hoc test.

opening of voltage-sensitive $Ca^{2+}$ channels. Also, $Ca^{2+}$ does not enter through NMDA receptors, because the $Mg^{2+}$ blockade is maintained under these conditions. Therefore, $Ca^{2+}$ entry is exclusively due to the opening of AMPA receptors lacking the GluR2 subunit. Stimulation of primary cell cultures with 100 $\mu$M KA in the presence of 30 $\mu$M CTZ, in NMG medium, significantly increased fluorescence above baseline in control cells (Fig. 6). The fluorescence of cells exposed to high glucose was significantly decreased compared with control cells. This observation suggests that the AMPA receptors present in retinal neural cells incubated with high glucose were less permeable to $Ca^{2+}$. In mannitol-treated cells, the $[Ca^{2+}]_i$ changes evoked by 100 $\mu$M KA in the presence of 30 $\mu$M CTZ were similar to the control.

## DISCUSSION

In this study, we found that elevated concentrations of glucose induce changes in the protein levels of several

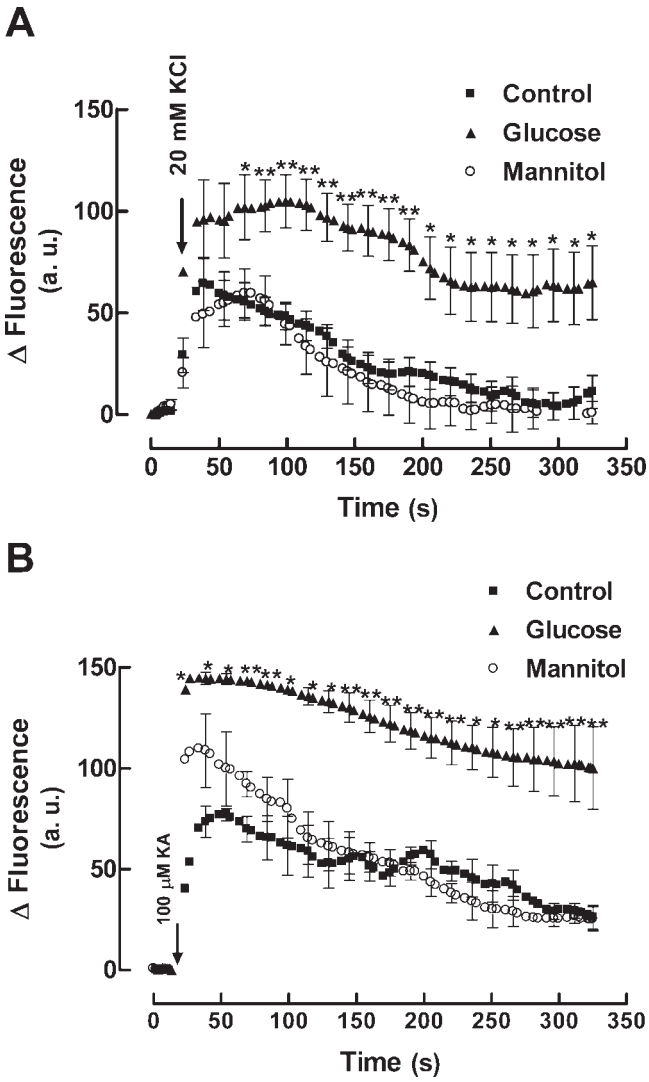


**FIGURE 5.** High glucose increased the $[Ca^{2+}]_i$ response evoked by KCl and kainate in primary cultures of neural cells from rat retina. Rat retinal cells were grown in 5 mM glucose (Control), 30 mM glucose (Glucose), and 25 mM mannitol plus 5 mM glucose (Mannitol) for 7 days. The cells were loaded with fluo-4/AM for 20 minutes, and changes in fluorescence were recorded by live-cell confocal microscopy. (**A**) After stimulation with 20 mM KCl the fluorescence in control and mannitol-treated cells increased transiently and returned to baseline, but the increase in fluorescence was significantly greater in high-glucose–treated cells and did not return to baseline for at least 300 seconds. (**B**) Similar results were obtained after stimulation with 100 $\mu$M KA. Results are presented as the mean ± SEM of at least five independent experiments and represent changes in arbitrary fluorescence units over basal fluorescence, recorded before stimulus application. $^*P < 0.05$, $^{**}P < 0.01$ significantly different from control; one-way ANOVA followed by the Dunnett post hoc test.

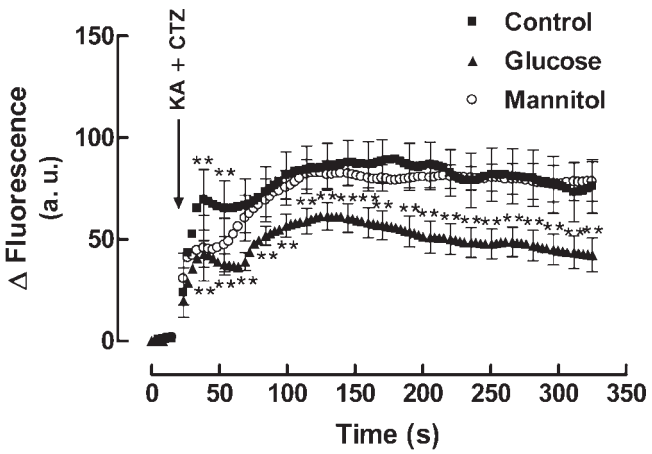


**FIGURE 6.** High glucose decreased the $[Ca^{2+}]_i$ response evoked by kainate in a $Na^+$-free medium, in primary cultures of neural cells from rat retina. Rat retinal cells were cultured in 5 mM glucose (Control), 30 mM glucose (Glucose), and 25 mM mannitol plus 5 mM glucose (Mannitol), for 7 days. The cells were loaded with fluo-4/AM for 20 minutes and stimulated with 100 $\mu$M KA plus 30 $\mu$M CTZ (to prevent AMPA receptor desensitization), in $Na^+$-free medium, where $Na^+$ ions are substituted by NMG. Changes in intracellular fluorescence were recorded by live-cell confocal microscopy. The increase in intracellular fluorescence was significantly less in high-glucose–treated cells compared to the control. Results are presented as the mean ± SEM of results in at least four independent experiments and represent changes in arbitrary fluorescence units over basal fluorescence, recorded before stimulation. $^{**}P < 0.01$ significantly different from control; one-way ANOVA followed by the Dunnett post hoc test.

ionotropic glutamate receptor subunits and alter the $Ca^{2+}$ homeostasis in cultured retinal neural cells. These effects were not due to increases in osmolarity, because equal concentrations of mannitol had no significant effect compared with the control.

AMPA and kainate receptors are important mediators of fast excitatory neurotransmission in the retina. In high-glucose–treated cell cultures, the protein content of GluR1 and GluR6/7 subunits decreased, whereas that of the GluR2 and KA2 receptor subunits increased. The expression of NMDA receptor subunits and the binding properties of AMPA receptors are also altered in the brain of diabetic rats, but the levels of GluR1 and GluR2/3 receptor subunits were unchanged.[36–38] More recently, it was reported that the NR1 and GluR2/3 subunits are more abundant in the retina of diabetic rats.[25] In our in vitro model, the changes in the content of ionotropic glutamate receptor subunits were not restricted to one type of receptor, since levels of both AMPA and kainate receptor subunits were altered by high glucose. The higher content of the GluR2 subunit is particularly significant because it decreases the $Ca^{2+}$-permeability of AMPA receptor-associated channels.

Cobalt staining was used as a functional indicator of the $Ca^{2+}$-permeability of the channel associated with AMPA receptors, because cobalt only enters cells through AMPA receptors lacking the GluR2 subunit.[39] Despite reports that kainate receptors may also mediate increases in intracellular $Ca^{2+}$,[33] in our hands, kainate receptors were not involved in the influx of cobalt. High glucose decreased the number of cells and the length of processes stained with cobalt, indicating a decrease in the number of $Ca^{2+}$-permeable AMPA receptors, consistent with the increase in the protein content of the GluR2 subunit. Overall, the morphology of retinal neurons was not altered by high glucose, as observed by MAP2 immunoreactivity, suggesting that the decrease in the number of cell bodies and the length of the processes stained with cobalt in high-glucose–treated cells was not due to a widespread change in morphology but to a reduction in cobalt uptake.

On depolarization, voltage-sensitive $Ca^{2+}$ channels are the main route for $Ca^{2+}$ entry into the cells. In this study, the increase in $[Ca^{2+}]_i$ evoked by membrane depolarization or AMPA receptor activation was greater in high-glucose–treated cells than in control or mannitol-treated cells. This effect may be due to an in increase in the abundance of voltage-sensitive $Ca^{2+}$ channels. Diabetes increases the density of $Ca^{2+}$ channels in other tissues (reviewed by Levy[40]). In dorsal root ganglion neurons from diabetic Bio Bred/Worchester (BB/W) rats there was an enhancement of high-voltage–activated currents, which increased with duration of diabetes up to 6 months.[41] Similar results were found in skeletal muscle from 8-week STZ-diabetic rats.[42] In addition, the $[Ca^{2+}]_i$ in cells treated with elevated concentrations of glucose did not recover to basal levels throughout the experiment, consistent with similar data from dorsal horn neurons of STZ-diabetic rats.[24] The delay in recovery of $[Ca^{2+}]_i$ to baseline suggests that the mechanisms responsible for removing the $[Ca^{2+}]_i$ are also affected by high glucose.

*N*-methyl-D-glucamine (NMG) has been widely used as a $Na^+$ substitute to maintain physiological solutions as isotonic without $Na^+$. This molecule does not enter through ion channels and therefore does not cause cell depolarization. In NMG medium, the voltage-sensitive $Ca^{2+}$ channels and NMDA receptors do not open, and $Ca^{2+}$ entry occurs essentially through AMPA receptors lacking the GluR2 subunit. Therefore, the influx of calcium specifically through AMPA receptors was examined by stimulating neural cells with kainate, in the presence of cyclothiazide to prevent desensitization of AMPA receptors. High glucose reduced the kainate-evoked $[Ca^{2+}]_i$ in NMG medium, compared with the control. This observation is consistent with the increase in the content of the GluR2 subunit and provides evidence that high glucose alters the function of AMPA receptors.

The combination of increased $Ca^{2+}$ influx and decreased $Ca^{2+}$ efflux can lead to sustained elevation in basal $[Ca^{2+}]_i$, which may trigger apoptosis.[19] The increase in the abundance of the GluR2 AMPA subunit due to elevated glucose may therefore be a compensatory protective mechanism in response to intracellular $Ca^{2+}$ overload. The increase in the $Ca^{2+}$-binding proteins calbindin and parvalbumin in the retina of diabetic rats is consistent with this possibility.[25]

Taken together, these results suggest that elevated glucose alters the content of ionotropic glutamate receptor subunits and $Ca^{2+}$ homeostasis in retinal neurons. This effect is likely to have an impact on normal function in the retina and may explain increased neuronal apoptosis and alterations in retinal function in diabetes. The alterations in $Ca^{2+}$ homeostasis found in retinal neurons may also play a role in the electroretinogram abnormalities induced by diabetes,[43] as well as in the reduction in contrast and color sensitivity recorded in humans with diabetes.[3–5]

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